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Original Article

The Effect of Rootstocks on the Sugars, Acids, Carotenoids, Chlorophylls and Ethylene of Clementine Mandarin (*Citrus clementina*)

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Abstract

Investigations have demonstrated that ratio of sugars to organic acids affects the particular taste of citrus fruit and carotenoids are essential for prevent of the disease. It seems that some rootstocks enhance the ripening of fruits and stimulate the biosynthesis of sugars and carotenoids. The aim of this study was to evaluate the effect of rootstocks on sugars, organic acids and carotenoids of fruit. The content of individual sugars and organic acids in fruits were determined by HPLC. Total acidity (TA), total soluble solids (TSS) and pH value of juice was also evaluated. Total carotenoid and chlorophylls content was measured using a spectrophotometer. The content of ethylene in fruits was determined by Gas chromatograph. HPLC analysis of juice allowed to detection of 3 sugars and 2 acids. Sucrose was the dominant sugar for all rootstocks. The amount of total sugars ranged from 102.27 (mg/ml) (Flying dragon) to 118.07 (mg/ml) (Orlando tangelo). The amount of total acids changed from 6.62 (mg/ml) (Trifoliate orange) to 8.48 (mg/ml) (Flying dragon). The amount of ascorbic acid varied from 0.44 (mg/ml) (Trifoliate orange) to 0.68(mg/ml) (Orlando tangelo).The pH value ranged from 3.65 (Flying dragon) to 3.95 (Orlando tangelo), TSS content changed from11.2 (%) (Flying dragon) to11.9 (%) (Orlando tangelo), TSS/TA varied from15.13 (Flying dragon) to 20.88(Orlando tangelo). Juice content ranged from 49.29 (%) (Trifoliate orange) to 54.47 (%) (Orlando tangelo). The amount of fruit production changed from 12 (Kg /tree) (Flying dragon) to 83(Kg /tree) (Orlando tangelo). The amount of total carotenoid varied from 0.12 (Murcott) to 0.15(Orlando tangelo, Sour orange, Flying dragon) (mg/gr DW). Among the six rootstocks evaluated, Orlando tangelo demonstrated the maximum rate of sugars, pH, TSS, TSS/TA, juice, ascorbic acid and carotenoids. As an outcome of our investigation, we can express that the rootstocks can affect the amount of sugars, acids and carotenoids of fruit.

Keywords: Carotenoids, Citrus rootstocks, Ethylene, Organic acids, Sugars

Abbreviations: HPLC, High performance liquid chromatography.

Introduction

Clementine mandarin (*Citrus .clementina*) is one of the most important mandarins are widely cultivated in Iran. Although it is an important crop, little research has been done on individual sugars, acids and carotenoids of Clementine mandarin.
Sugars of citrus fruit have been classified into three major categories: fructose, glucose and sucrose. Sucrose is known as the dominant sugar in citrus fruit and is plentiful. Sugars usually display 80% of the total soluble solids of juice [1]. Soluble solids are mixture of organic acids and sugars that applied as an index of maturity and taste quality [2]. Ascorbic acid is an antioxidant and exhibits a key function in the reduction of diseases. Carotenoids are also known to reduce cancers, cataracts, and heart disease [3]. Carotenoids are also widely used in the foodstuff, cosmetic and medicine products as natural coloring agent [4]. In Citrus fruits, ethylene will be

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able to stimulate ripening by increasing the biosynthesis of carotenoids and chlorophylls destruction [5].

Ratio of sugars to acids affects the flavor of citrus fruit and has been considered as quality indicator by both fresh consumption group and juice factories [6]. Citrus juice is a fantastic resource of sugars and acids. The amount of citrus sugars is changeable and is dependent on the rootstock [7], cultivar [8] and etc. A number of researches have indicated that the rootstocks can influence the sugars and acids of citrus fruit [9,10]. The aim of this research is to identify rootstock that can synthesize the maximum level of sugars and carotenoids.

Method and Materials

Chemicals and Standards

Fructose, glucose, sucrose, ascorbic acid, citric acid, ethylene standards, acetonitrile, butylated hydroxytoluene (BHT) and diethyldithiocarbamate (DDC) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide and phosphoric acid were purchased from Merck (Darmstadt, Germany).

Rootstocks

In 2001, rootstocks were planted at 8×4 m with three replication at Ramsar research station [Latitude 36° 54' N, longitude 50° 40' E; Caspian Sea climate, average rainfall and temperature were 970 mm and 16.25˚C per year respectively; soil was classified as loam-clay, pH ranged from 6.9 to 7]. Sour orange, Swingle citrumelo, Trifoliate orange, Flying dragon, Orlando tangelo and Murcott were used as rootstocks in this experiment (Table 1).

Preparation of Juice Sample

Fruits were collected from different parts of the same trees in January 2016, early in the morning (6 to 8 am) and only during dry weather. The selection method was on the basis of completely randomized design. Fruits juice was extracted using juicer. Then, Juices were centrifuged at 15,000 rpm for 20 min at 4 ˚C. Three replicates were done for this research (n=3) [9]

Juice Analyses Technique

The total titratable acid was determined by titration with sodium hydroxide (0.1 N) and displayed as % citric acid. Total soluble solids were measured using a refractometer (Kruss, Germany). The pH value was determined using a digital pH meter (Jenway, Model: 3510). Sugars, citric acid and ascorbic acid were measured by HPLC [9].

Analysis of Sugars by HPLC

HPLC analysis was performed with a PLATIN blue system (Knauer, Berlin, Germany) equipped with binary pump and a Refractive Index (RI) detector. The separation was carried out on a Shodex Asahipak NH2P-50 4E column. Column temperature was maintained at 25 ˚C, and the injection volume for all samples was 10 μL. Elution was performed isocratically with the mobile phase consisting of 75% (v/v) acetonitrile (eluent A) and 25% (v/v) water (eluent B) at a flow rate of 1 mL/ min. Identification of sugars was based on retention times of unknown peaks in comparison with standards. The concentration of the sugars was calculated from peak area according to calibration curves. Standard solutions of sugars (fructose, glucose and sucrose) and organic acids (ascorbic acid and citric acid) were prepared by dissolving the required amount of each standard in deionized water.

Table 1 Common and botanical names for citrus taxa used as rootstocks and scion.

Common name	Botanical name	Parents	Category
Clementine (scion)	*Citrus clementina cv. Cadox*	Unknown	Mandarin
Sour orange (Rootstock)	*Citrus × aurantium*	Mandarin×Pomelo	Sour orange
Swingle citrumelo (Rootstock)	*Swingle citrumelo*	*C.paradisi cv. Duncan ×P.trifoliata (L.) Raf*	Poncirus hybrids
Trifoliate orange (Rootstock)	*Poncirus trifoliata (L.) Raf*	Unknown	Poncirus
Flying dragon (Rootstock)	*Poncirus trifoliata (L.) Raf*	Unknown	Poncirus
Orlando tangelo (Rootstock)	*Citrus sp. cv. Orlando*	*Citrus reticulata cv. Dancy×Citrus paradisi cv.Duncan*	Tangelo
Murcott (Rootstock)	*Citrus sp. cv. Murcot*	*C.reticulata× C.sinensis*	Tangor

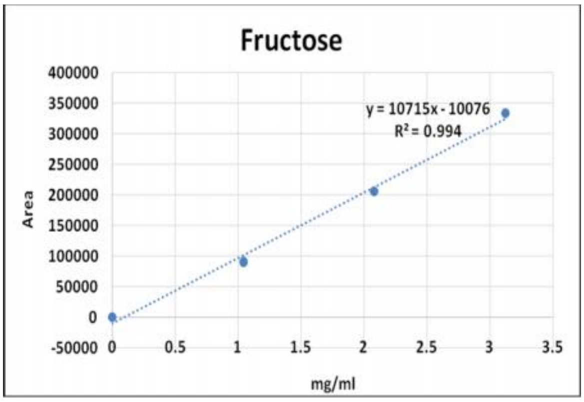

Fig. 1 The standard curve of fructose

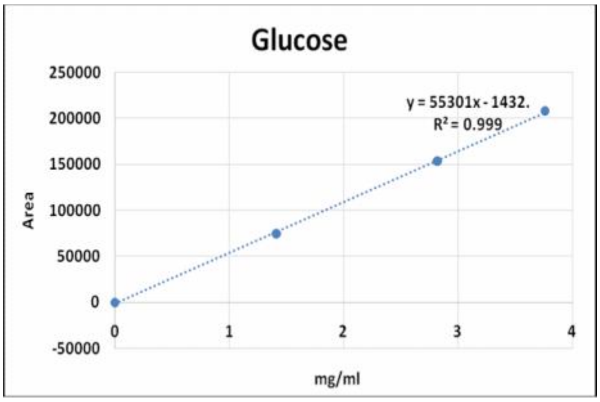

Fig. 2 The standard curve of glucose

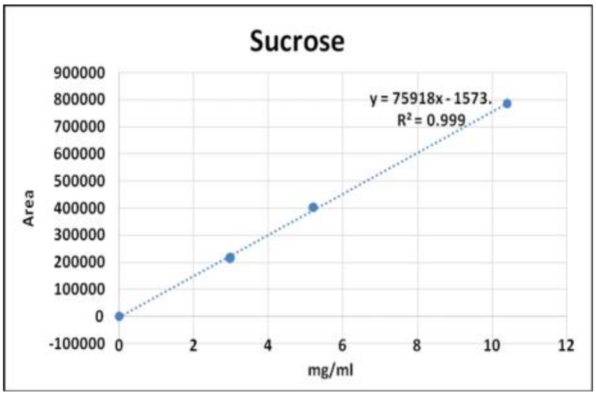

Fig. 3 The standard curve of sucrose

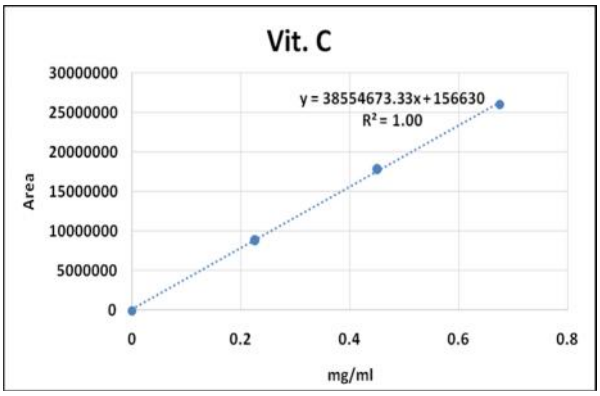

Fig. 4 The standard curve of ascorbic acid

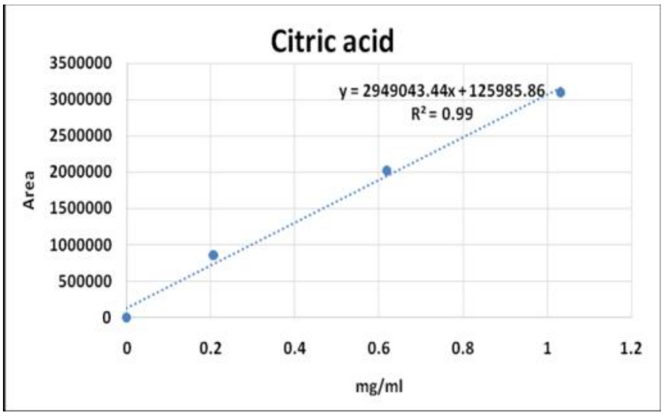

Fig. 5 The standard curve of citric acid

Calibration was performed by injecting the standard three times at four different concentrations. Standard solution of fructose at concentrations of 0, 1.04, 2.08 and 3.12 mg/mL, used to obtain a standard curve. Standard solutions of glucose at concentrations of 0, 1.41, 2.82 and 3.76 mg/mL, used to obtain a standard curve. Standard solutions of sucrose at concentrations of 0, 2.97, 5.20 and 10.40 mg/mL, used to obtain a standard curve. Standard solutions of ascorbic acid at concentrations of 0, 0.22, 0.45 and 0.67 mg/mL, used to obtain a standard curve. Standard solutions of citric acid at concentrations of 0, 0.20, 0.61 and 1.03 mg/mL, used to obtain a standard curve. (Fig. 1 to 5). Sugars concentration was estimated from calibration curve and the result was expressed as milligrams of compound per milliliter (mg/ mL).

Analysis of Acids by HPLC

A same HPLC was applied for this study. It fitted with an ODS-2 C-18 reversed phase column and a photodiode array (PDA) detector. The column temperature was set on 25 ˚C. Elution was performed isocratically with the mobile phase consisting of

0.05% (v/v) aqueous phosphoric acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.6 mL/ min. Chromatograms were recorded at 254 nm for citric acid and ascorbic acid. Acids concentration was estimated from calibration curve and the result was displayed as milligrams of compound per milliliter (mg/ mL).

Identification of Sugars and Acids Components

Sugars and organic acids were identified by comparing the retention times with those of known.

Determination of Total Carotenoids and Chlorophylls

The method applied in this study, was explained by Van-Wyka *et al* [11]. Peels were freeze-dried at –56 ˚C for 4 days to lose all their moisture and then powdered by a mill. Samples were frozen at –80 ˚C until analyzed. All extractions were carried out under low light conditions to decrease of photo destruction. Briefly, 0.2 g freeze-dried sample was mixed with 10mL of ethanol solvent (95% v/v), butylated hydroxytoluene (BHT) (100 mg L^{-1}) and diethyldithiocarbamate(DDC) (200 mg L^{-1}). The samples were inverted for two min and kept at 4 ˚C. The samples were passed through an ashless filter paper. The filtrates were putted in a spectrophotometer (UV 1600 PC,Shimadzu, Tokyo, Japan) and absorbances were determined at 470 nm, 649 nm and 664 nm. The concentration of chlorophylls and total carotenoid were calculated by the following formula. Results were displayed as mg of chlorophyll or carotenoid per g dry weight (mg g^{-1} dry weight).

$$C_a = \frac{(13.36A_{664.2} - 5.19A_{648.6})*8.1}{DW} \qquad [mg\,g^{-1}dw]$$

$$C_b = \frac{(27.43A_{648.6} - 8.12A_{664.2})*8.1}{DW} \qquad [mg\,g^{-1}dw]$$

$$C_{a-b} - \frac{(5.24A_{664.2} + 22.24A_{648.6})*8.1}{DW} \qquad [mg\,g^{-1}dw]$$

$$C_{x-c} = \frac{(4.785A_{470} + 3.657A_{664.2} - 12.76A_{648.6})*8.1}{DW} \qquad [mg\,g^{-1}dw]$$

Chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophylls (C_{a+b}) and total carotenoids (C_{x+c}):

Ethylene Extraction Technique

In order to obtain the ethylene, fruits were weighed and were placed in a jar. The jar was covered and placed at room temperature for 1h. The temperature of room was holding constant at 25 ˚C. The volume of headspace around the fruits was measured. Ethylene was extracted with a 50mL plastic syringe through the septum of jar. Injection volume was 1 mL.

Analysis of Ethylene by GC

An Agilent 7890A gas chromatograph (USA) was applied for this study. It fitted with a HP-5 column. The column temperature was set on 70 ˚C. The injector temperature was set on 160 ˚C. The detector temperatures were set on 135 ˚C. Helium was applied as the carrier gas at a flow rate of 37 ml/min. Ethylene concentration was estimated from calibration curve and the result was displayed as nanoliter per kilogram fresh weight of fruit per hour (nL kg^{-1} h^{-1}).

Physical Traits of Fruit and Fruit Production (yield)

Fifty fruits were randomly sampled and evaluated for each tree. Fruit physical traits were presented in Table 2. Total dry matter was determined by dehumidify of fruits in an oven at 80 ˚C. Ash was measured by placing the weighed fruits in a furnace at 560 ˚C. The weight of fresh fruit was determined using a scale. The weight of dried fruit evaluated with oven. Fruit length, fruit diameter and rind thickness were determined using a caliper. Fruit shape index was explained as the ratio of fruit diameter to length. The fruit yield was measured separately for each tree. Fruits for each tree were measured using a digital scale.

Data Analysis

SPSS 18 was used for analysis of the data obtained from the experiments. Analysis of variations was based on the measurements of 24 traits. Comparisons were made using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences were considered to be significant at $P<0.01$. The correlation between pairs of characters was evaluated using Pearson's correlation coefficient.

Results

Result of the HPLC Analyses

HPLC analyses of juice allowed to identification of 3 sugars (fructose, glucose and sucrose) and 2 acids (citric acid and ascorbic acid) (Fig. 6, Fig. 7, Table 2).

Determination of Sugars

Fructose, glucose and sucrose were three sugars that recognized in this study. Moreover, the amount of total sugars ranged from 102.27 to 118.07 mg/ mL. Sucrose was the dominant sugar in this study. Among six rootstocks evaluated, Orlando tangelo indicated the maximum level of sugars (Table 2).

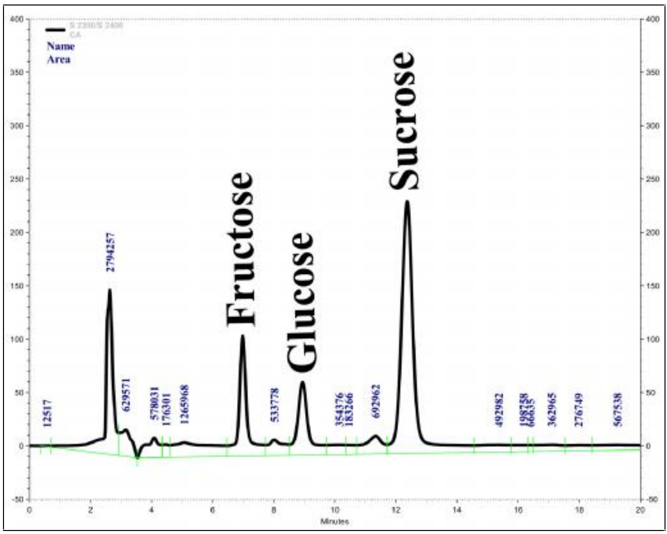

Fig. 6 HPLC chromatogram of sugars of Clementine mandarin

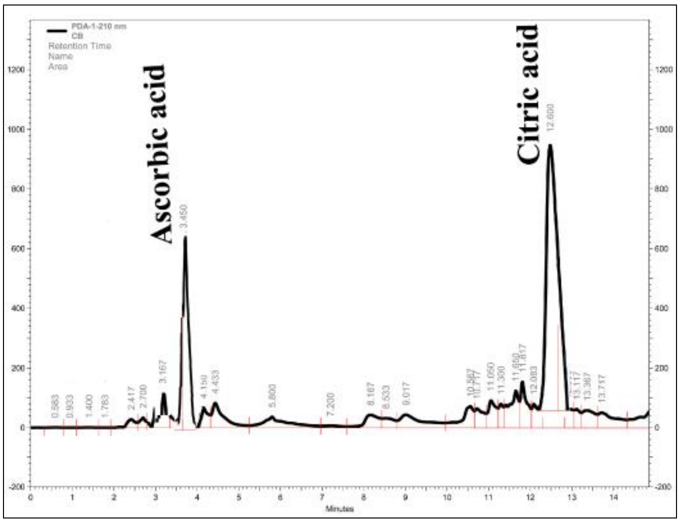

Fig. 7 HPLC chromatogram of acids of Clementine mandarin

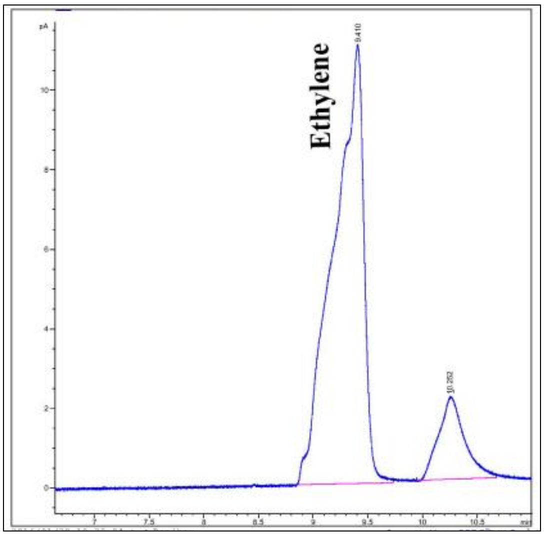

Fig. 8 GC ethylene chromatogram of Clementine mandarin

Table 2 Statistical analysis of variation in juice compositions and fruit physical traits of Clementine mandarin on six different rootstocks.

Compounds	Sour orange		Swingle citrumelo		Trifoliate orange		Flying Dragon		Orlando tangelo		Murcott		
	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	Mean	St.err	F value
Sugars	-	-	-	-	-	-	-	-	-	-	-	-	-
1) Fructose (mg/ml)	13.71 b	0.20	12.39 c	0.24	1.42 e	0.12	10.91 d	0.12	14.51 a	0.25	11.28 d	0.28	**
2) Glucose (mg/ml)	20.55 b	0.25	19.88 c	0.17	20.91 b	0.21	17.59 d	0.19	22.53 a	0.25	17.59 d	0.17	**
3) Sucrose (mg/ml)	79.14 b	0.26	73.78 d	0.20	80.43 a	0.30	73.77 d	0.22	81.03 a	0.26	75.56 c	0.23	**
Total	113.4	0.71	106.05	0.61	102.76	0.63	102.27	0.53	118.07	0.76	104.43	0.68	-
Organic acids	-	-	-	-	-	-	-	-	-	-		-	-
1) Citric acid (mg/ml)	7.74 a	0.24	7.28 ab	0.18	6.18 d	0.22	7.86 a	0.25	6.95 b	0.20	6.26 d	0.26	**
2)Ascorbic acid (mg/ml)	0.64 ab	0.04	0.54 bc	0.04	0.44 c	0.05	0.62 ab	0.03	0.68 a	0.04	0.46 c	0.05	**
Total	8.38	0.28	7.82	0.22	6.62	0.27	8.48	0.28	7.63	0.24	6.72	0.31	
Total titratable acid (%)	0.70 b	0.01	0.69 b	0.03	0.61 c	0.01	0.74 a	0.01	0.57 c	0.006	0.69 b	0.006	**
pH	3.67 b	0.10	3.69 b	0.10	3.75 b	0	3.65 b	0.10	3.95 a	0.05	3.70 b	0.05	**
TSS (%)	11.8 ab	0.30	11.7 b	0.20	11.3 c	0.30	11.2 c	0	11.9 a	0.20	11.5 abc	0.10	**
TSS/TA	16.86 c	0.22	16.95 c	0.44	18.52 b	0.30	15.13 d	0.26	20.88 a	0.20	16.66 c	0.35	**
Juice (%)	53.48 b	0.28	53.22 b	0.11	49.29 d	0.13	49.78 d	0.23	54.47 a	0.14	51.84 c	0.18	**
Total dry matter (%)	13.70 b	0.22	14.80 a	0.11	13.24 bc	0.39	13.64 b	0.86	13.24 bc	0.14	12.59 c	0.07	**
Ash (%)	4 a	0.00	4 a	0.00	3 b	0.00	3 b	0.00	4 a	0.00	3 b	0.00	**
Fresh fruit weight (g)	80.34 ab	5.00	85.12 ab	5.55	77.92 b	5.84	74.32 b	4.44	93.36 a	5.44	79.93 ab	4.38	**
Dry fruit weight[z] (g)	8.22 b	0.14	8.88 a	0.12	7.94 bc	0.23	8.18 b	0.09	7.94 bc	0.19	7.55 c	0.20	**
Fruit diameter (mm)	55.00 c	2.10	57.81 bc	3.00	52.00 c	2.90	50.80 c	2.00	62.50 a	3.30	53.80 c	2.40	**
Fruit length (mm)	46.61 c	2.50	53.52 b	2.30	48.59 bc	2.80	46.18 c	2.00	59.52 a	2.10	48.46 bc	2.30	**
Fruit shape index (Fd/Fl)	1.18 a	0.02	1.08 b	0.03	1.07 b	0.02	1.10 b	0.03	1.05 b	0.03	1.11 b	0.02	**
Rind fruit weight[z] (g)	12.58 b	0.86	12.58 b	1.26	15.38 ab	1.60	16.35 a	0.10	13.00 b	0.75	15.50 ab	1.73	**
Rind thickness (mm)	2.0 b	0.1	2.2 b	0.1	2.5 a	0.1	2.7 a	0.1	2.2 b	0.1	2.6 a	0.1	**
Fruit production (Kg /tree)	71 b	5	75 ab	4	51 c	4	12 d	2	83 a	5	71b	4	**
Carotenoids (mg/gr DW)	0.15 a	0.01	0.13 bc	0.01	0.14 ab	0.01	0.15 a	0.01	0.15 a	0.01	0.12 c	0.01	**
Chlorophyl A(mg/gr DW)	0.001 c	0.00	0.00	0.00	0.05 a	0.01	0.00	0.00	0.004 b	0.00	0.002 c	0.00	**
Chorophyl B(mg/gr DW)	0.004 c	0.00	0.00	0.00	0.02a	0.00	0.00	0.00	0.01 b	0.00	0.001 d	0.00	**
Total chrophyl(mg/grDW)	0.005 c	0.00	0.00	0.00	0.07 a	0.01	0.00	0.00	0.01 b	0.00	0.003 c	0.00	**
Ethylene (nL kg^{-1} h^{-1})	27 b	3	22 c	2	22 c	2	36.5 a	3	12. 5 d	1	11.5 d	1	**

Mean is average of traits used with three replicates. St. err = standard error. Results of analysis of variance: ** significant difference at $P \leq 0.01$. Any two means within a row not followed by the same letter are significantly different at $P \leq 0.01$. [z] For 60g fruit.

Table 3 intercorrellations between 6 compositions in a correlation matrix

	Fructose	Glucose	Sucrose	Citric acid
Glucose	0.01	-	-	-
Sucrose	-0.21	0.81**	-	-
Citric acid	-0.24	-0.09	-0.43	-
Ascorbic acid	0.12	0.21	-0.26	0.84**

*=significant at 0.05, **=significant at 0.01

Results of Total Titratable aAcid (TA)

Determination of Organic Acids

Citric acid and ascorbic acid were two acids that recognized in this study. Moreover, the amount of total acids ranged from 6.62 to 8.48 mg/ mL. Among six rootstocks evaluated, Flying dragon indicated the maximum level of acids (Table 2).
The amount of total titratable acid ranged from 0.57 to 0.74%. Among six rootstocks evaluated, Flying dragon indicated the maximum level of total acidity (Table 2).

Results of pH, TSS, TSS/TA and Juice Content

The amount of pH, TSS, TSS/TA and juice content were given in Table 2. Among six rootstocks evaluated, Orlando tangelo indicated the maximum level of pH, TSS, TSS/TA and juice content.

Results of Fruit Physical Traits and Fruit Production (Yield)

The amount of fruit physical traits and fruit production were given in Table 2. Among six rootstocks evaluated, Orlando tangelo indicated the maximum level of fresh fruit weight, fruit diameter, fruit length and fruit production.

Determinations of Total Carotenoids and Chlorophylls Contents

The amount of total carotenoids and chlorophylls were given in Table 2. Among six rootstocks evaluated, Orlando tangelo, Sour orange and Flying dragon indicated the maximum level of total carotenoids.

Result of the Ethylene Analysis

GC analyses of fruits allowed identification of ethylene in retention time of 9.41 minutes (Fig. 8). Among six rootstocks evaluated, Flying dragon indicated the maximum level of ethylene (Table 2).

Statistical Analyses

Differences were considered to be significant at $P < 0.01$. These differences on the 1% level occurred in fructose, glucose, sucrose, citric acid, ascorbic acid, TA, pH, TSS, TSS/TA , juice, total dry matter , ash , fresh fruit weight, dry fruit weight, fruit diameter, fruit length , fruit shape index , rind fruit weight, rind thickness , fruit production , carotenoids, chlorophyll A, chlorophyll B, Total chlorophyll and ethylene (Table 2).

Results of Correlation

Sucrose and glucose demonstrated a significant positive correlation with each other. Ascorbic acid and citric acid also demonstrated a significant positive correlation with each other (Table 3).

Discussion

The results indicated that the sugars and organic acids were affected by rootstocks. These findings were consistent with the results of other researchers [9]. The compositions of sugars obtained from six rootstocks of Clementine mandarin were very similar. However, the relative concentration of compounds was different according to the type of rootstock. Comparison of our data with those in the literatures revealed that the amount of sugars and acids were not consistent with previous studies [9]. It might be related to rootstock, ecological and environmental factors that could influence the compositions. However, it should be noted that the extraction method might also affected the results. Studies showed that fertilizer and irrigation were affected the content of sugars present in crops [12]. Fertilization, irrigation and other operations were carried out uniform in this study so we did not believe that these variations might be due to the variation in environmental conditions
The discovery of sucrose -6- phosphate, as an intermediate between UDP- Glucose and sucrose, led to a rapid description of the biosynthetic pathway of sugar compounds. The biosynthetic pathway of sugar compounds in higher plants is as follows:
Photosynthesis →Triose-P → Fructos- 6-phosphate→ Glucose- 6- phosphate→ Glucose- 1-

phosphate→UDP- Glucose→ Sucrose -6- phosphate →Sucrose → Glucose and Fructose[13].

Reaction pathway catalyzed by sucrose-6-phosphate synthase and sucrose-6-phosphate phosphatase respectively [14]. An increase in the amount of sugars, when Orlando tangelo, used as the rootstock, showed that either the synthesis of Triose-Ps was enhanced or activities of both enzymes increased.

Studies have shown that plant hormones affect sugars of fruit [15]. On the other hand, the level of plant hormones can also be changed by rootstocks [16].

Research has shown that ethylene can stimulate the biosynthesis of carotenoids and can reduce chlorophylls of citrus peel [5]**.** On the other hand, the level of ethylene can also be changed by rootstocks [16].

It is commonly accepted that carotenoids in higher plants are originated from acetyl-CoA via the mevalonic acid pathway [Acetyl-CoA →Mevalonic acid→ Geranylgeranylpyrophosphate → Phytoene→Lycopene→ α-caroten or β-caroten] [17].

Considering that Triose-P is necessary for the synthesis of sugars, it can be assumed that there is a specialized function for this molecule and it may be better served by Orlando tangelo.

Conclusion

In the present study we found that the amount of sugars and acids were significantly impressed by rootstocks and there was a great variation in most of the measured characters among six rootstocks. The present study demonstrated that the relative concentration of sugars and acids was different according to the type of rootstock. Among six rootstocks examined, Orlando tangelo showed the highest content of sugars, pH, TSS, TSS/TA, juice, ascorbic acid and carotenoids. The lowest of sugars, pH, TSS, TSS/TA and juice content were produced by Flying dragon. Further research on the relationship between rootstocks and sugars is necessary.

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